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Establishment of Mammary Tumors by Injection of 7,12-Dimethylbenz[a]anthracene in Mammary Fat Pad of Rats

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ABSTRACT

Chemical-induced mammary cancer models are widely used to mimic human breast carcinogenesis, with 7,12-dimethylbenz[a]anthracene (DMBA) being a commonly used agent. The oral administration of DMBA frequently results in the formation of tumors at random locations and carries significant risks, including high mortality rates and damage to various organs. To address these issues, this study employed a subcutaneous DMBA administration protocol to induce mammary cancer in rats. A total of twenty-four female Sprague-Dawley rats aged 45-55 days weighing 112-130 g were divided into four groups, including the control group injected with 0.75 mL corn oil (D0), a single dose of DMBA at 80 mg/kg BW (D1), two doses with a one-week interval (D2), and three doses with one-week intervals (D3), all administered via subcutaneous in mammary fat pad. Control groups (D0) did not show any tumor growth. Mammary tumor incidence increased with dosage (D1 33.33%, D2 66.67%, and D3 100%). Histopathological examination revealed the presence of various mammary tumor types without evidence of metastasis in all induced rats. All tumors originated from the injection site, and only a single nodule was observed in each rat. There were no significant differences in tumor grades between the treatment groups, and no mortality was recorded during the study. The D3 group showed the highest tumor incidence over the three-month observation period. These findings suggest that subcutaneous DMBA administration effectively induces mammary cancer in rat models with controlled tumor localization and minimal systemic effects, making it a promising method for experimental breast cancer studies.

Keywords: 7,12-dimethylbenz[a]anthracene, Animal model, Breast cancer, Mammary fat pad, Rat

INTRODUCTION

Chemical-induced mammary cancer models are widely used to simulate human breast cancer carcinogenesis, effectively representing the associated cellular and molecular changes (Abba et al., 2016) compared to tumor-transplanted models (Costa et al., 2020). A variety of chemical compounds, such as 7,12-dimethylbenz[a]anthracene (DMBA), N-methyl-N-nitrosourea (NMU), 2-amino-1-methyl-6-phenylimidazo[4,5-B] pyridine (PhIP3), and methylcholanthrene (MC), have been demonstrated to induce mammary cancer in animal models (Bazm et al., 2018). Among these, DMBA, a member of the polycyclic aromatic hydrocarbon (PAH) family, is frequently employed due to its ability to mimic the multistep carcinogenic process observed in human breast cancer (Liu et al., 2015).

Significant efforts have been made to develop breast cancer models, with studies examining various doses and administration routes of DMBA. Oral administration is the most common but has notable drawbacks, including high mortality (Fidianingsih et al., 2022), pathological lesions in multiple organs (Budi and Widyarini, 2010; Batcioglu et al., 2012; Costa et al., 2020; Allam et al., 2023), and risks of acute death from lung exposure due to administration errors. Additionally, oral DMBA induces randomly located tumors with varying nodule numbers (Barros et al., 2004; Costa et al., 2020), complicating studies requiring a single, well-defined nodule for targeted therapy. Systemic administration can also lead to tumors in mammary glands and other tissues (Dias et al., 1999; Tedardi et al., 2013).

To address these challenges, subcutaneous administration of DMBA into the mammary fat pad has been proposed, enabling localized tumor formation while minimizing the systemic effects and mortality. Several studies have employed this approach with varying doses (El-Makawy et al., 2022; Silihe et al., 2023; Sewoyo et al., 2024). However, detailed histopathological characteristics and their systemic effect remain underreported. The present study aimed to evaluate the

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mortality rate, and detailed histopathological characteristics of mammary cancer induced by subcutaneous administration of DMBA, comparing single dose versus repeated doses and evaluating its systemic effect on internal organs. In addition, the study aimed to provide a clearer understanding of the impact of this method on tumor localization and grading while minimizing systemic effects that could influence experimental outcomes.

MATERIALS AND METHODS

Ethical approval

The current study received ethical permission from the Animal Testing Ethics Committee of the Veterinary Medicine Faculty, Udayana University, Indonesia (No. B/145/UN14.2.9/PT.01.04/2024). All procedures were conducted in accordance with the strict guidelines and regulations of the committee.

Animals

Twenty-four nulliparous female Sprague Dawley rats (*Rattus norvegicus*), aged 45-55 days and weighing between 112-130 g, were used as experimental subjects. The animals were obtained from the Department of Pharmacology, Faculty of Medicine, Udayana University, Indonesia. The rats in the four treatment groups were housed in separate cages, each measuring 53 x 38 x 16 cm, in the Pathobiology Laboratory, Faculty of Veterinary Medicine, Udayana University, under a 12-h light/12-h dark cycle. The rats were provided with standard pellet feed and water *ad libitum*.

7,12-dimethylbenz[a]anthracene preparation

The rats were weighed to determine their body weight, and the corresponding DMBA dose was calculated (El-Makawy et al., 2022). The DMBA (Tokyo Chemical Industry, Tokyo, Japan) was weighed according to the calculated dose and dissolved in 0.75 mL of corn oil using a magnetic stirrer until the solution was homogeneous. An autoclave sterilized the solution (Sewoyo et al., 2024).

Treatment protocols

Before treatment, the rats were acclimatized for two weeks. The rats were divided into four groups including D0 received a single injection of vehicle (corn oil) 0.75 mL, D1 received a single injection of DMBA at 80 mg/kg BW, D2 who received two injections with one-week intervals, and D3 received three injections, also with one-week interval. Each treatment group consisted of six rats that were randomly allocated to each group. Before each injection, the rats were anesthetized with ketamine and xylazine at doses of 75 mg/kg BW and 10 mg/kg BW respectively, by i.m. injection (IACUC, 2011). The injection site was shaved with clippers, followed by aseptic preparation. DMBA was administered subcutaneously into the mammary fat pad. The study period lasted three months, during which tumor growth was monitored. Rats were euthanized when tumor masses exceeded 30 mm in diameter or when they exhibited poor general health.

Euthanasia and sample processing

Rats were euthanized by intraperitoneal injection of a lethal dose (10 times the normal dose) of ketamine and xylazine according to American Veterinary Medical Association (AVMA) guidelines (Leary et al., 2020). After rats were euthanized, tumor tissue was excised. Rats were also necropsied to collect internal organs, such as lungs, heart, liver, kidney, and stomach. All tissues were fixed in 10% neutral buffered formaldehyde for 24 hours, followed by routine histological preparation, and stained with Harris hematoxylin-eosin.

Histopathological examination

The histopathological characteristics of the tumors were evaluated based on Goldschmidt et al. (2011) and do Nascimento and Otoni (2020). Tumor grading was conducted according to the Nottingham grading system (Elston and Ellis, 1991). Multiple organs were evaluated for pathologic lesions and neoplastic transformation.

Data analysis

Histopathological types and tumor grades are presented in tabular form, while microscopic findings are described narratively. Differences in tumor incidence and histopathological grade between groups were assessed through the Kruskal-Wallis test and Mann-Whitney U-test. Statistical analyses were performed using SPSS version 26, 2018 for Windows, and a p-value < 0.05 was considered statistically significant.

RESULTS

Histopathology characterization and tumor grading

In this study, mammary tumors were induced via subcutaneous injections of DMBA into the mammary fat pad, and the effects of different dosing regimens were observed over three months. Tumor development and characteristics were subject to rigorous monitoring. The control group (D0), which received the vehicle solution (corn oil), did not develop any tumors (0%). In the D1 group, which received a single dose of DMBA, two out of six rats (33.33%) developed tumors. The D2 group, which received two doses, showed an increased tumor incidence, with four out of six rats (66.67%) developing tumors. The highest tumor incidence was observed in the D3 group, in which all six rats (100%) developed tumors after receiving three doses of DMBA.

The histopathological analysis revealed the tumor compositions in the D1 group. The tumors were found to consist of cribriform ductal carcinoma *in situ* (DCIS, 50%, Figure 1a) and papillary DCIS (50%, Figure 1b). The D2 group exhibited solid DCIS (25%, Figure 1c), cribriform DCIS (50%), and comedo DCIS (25%, Figure 1d). In the D3 group, a more diverse range of tumors was observed, including lipid-rich carcinoma (16.67%, Figure 1e), invasive cribriform carcinoma (16.67%, Figure 1f), comedo DCIS (33.33%), and cribriform DCIS (33.33%, Table 1).

The results revealed a statistically significant difference in tumor incidence between the groups ($p < 0.05$). The results confirmed a significant difference in tumor incidence between D0 and D2, D0 and D3, and D1 and D3 ($p < 0.05$). It can be concluded that DMBA injection significantly increased tumor incidence compared to the control group. Three repeated doses of 80 mg/kg of DMBA significantly increased tumor incidence compared to a single dose injection. Despite these differences in incidence, there was no significant variation in tumor grade across the dosing regimens as indicated by the Kruskal-Wallis test.

It is noteworthy that all tumors consistently originated at the injection site, with each rat developing only one tumor nodule. No mortality occurred during the study, and examination of internal organs, including the lungs, heart, kidneys, and stomach, revealed no evidence of tumor formation. The finding indicated that DMBA-induced tumors were localized specifically to the mammary glands. Moreover, no metastasis was detected throughout the three-month observation period.

In addition to the tumor formation, several organs in rats across all treatment groups exhibited mild lesions. These included pulmonary inflammation (Figure 2a), hepatic inflammation and vacuolar degeneration (Figure 2b), and renal inflammation and tubular degeneration (Figure 2c, 2d). No lesion was observed in the heart and stomach of any of the rats in all the treatment groups (Figure 2e, 2f). All organs exhibited no indications of toxicity. A comprehensive description of the lesions observed in each treatment group is presented in Table 2.

Table 1. Histopathological classification and tumor grading in rats administrated with 7,12- dimethylbenz[a]anthracene during a three-month observation period

Group	Rat no.	Histopathological type	Score*			Tumor grade
			Tubular Formation	Nuclear Atypia	Mitosis Count	
D1	1	Cribriform DCIS	2	1	1	1
	2	Papillary DCIS	2	2	2	2
	3	-	-	-	-	-
	4	-	-	-	-	-
	5	-	-	-	-	-
	6	-	-	-	-	-
D2	1	Solid DCIS	3	1	1	1
	2	Cribriform DCIS	3	2	2	2
	3	Cribriform DCIS	3	2	2	2
	4	Comedo DCIS	3	2	2	2
	5	-	-	-	-	-
	6	-	-	-	-	-
D3	1	Invasive Cribriform Carcinoma	3	3	3	3
	2	Comedo DCIS	3	2	2	2
	3	Comedo DCIS	3	2	2	2
	4	Lipid-rich Carcinoma	3	2	2	2
	5	Cribriform DCIS	3	2	1	2
	6	Cribriform DCIS	3	2	1	2

Note: D1 received a single injection of DMBA at 80 mg/kg BW, D2 received two injections with a one-week interval, and D3 received three injections, also with a one-week interval. DCIS: Ductal carcinoma *in situ*. *Score criteria are based on the Nottingham Grading System. Tubular formation, nuclear atypia, and mitosis count scores were tabulated. Score 3-5: Grade 1; Score 6-7: Grade 2; Score 8-9: Grade 3.

Table 2. Lesions in the internal organ of rats after induction of 7,12-dimethylbenz[a]anthracene

Group	Organ	Lesions				
		Deg.	**Infl.**	**Nec.**	**Met.**	**Neo.**
D0	Lungs	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)
	Heart	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)
	Liver	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)
	Kidney	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)
	Stomach	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)
D1	Lungs	(0/6)	(2/6)	(0/6)	(0/6)	(0/6)
	Heart	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)
	Liver	(1/6)	(3/6)	(0/6)	(0/6)	(0/6)
	Kidney	(1/6)	(2/6)	(0/6)	(0/6)	(0/6)
	Stomach	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)
D2	Lungs	(0/6)	(1/6)	(0/6)	(0/6)	(0/6)
	Heart	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)
	Liver	(2/6)	(2/6)	(0/6)	(0/6)	(0/6)
	Kidney	(2/6)	(1/6)	(0/6)	(0/6)	(0/6)
	Stomach	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)
D3	Lungs	(0/6)	(3/6)	(0/6)	(0/6)	(0/6)
	Heart	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)
	Liver	(1/6)	(2/6)	(1/6)	(0/6)	(0/6)
	Kidney	(2/6)	(2/6)	(0/6)	(0/6)	(0/6)
	Stomach	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)

Note: D0 received a single injection of vehicle (0.75 mL corn oil), D1 received a single injection of DMBA at 80 mg/kg BW, D2 received two injections with one-week intervals, and D3 received three injections, also at one-week intervals. Deg: Degeneration; Infl: Inflammation; Nec: Necrosis; Met: Metastasis; Neo: Neoplasia.

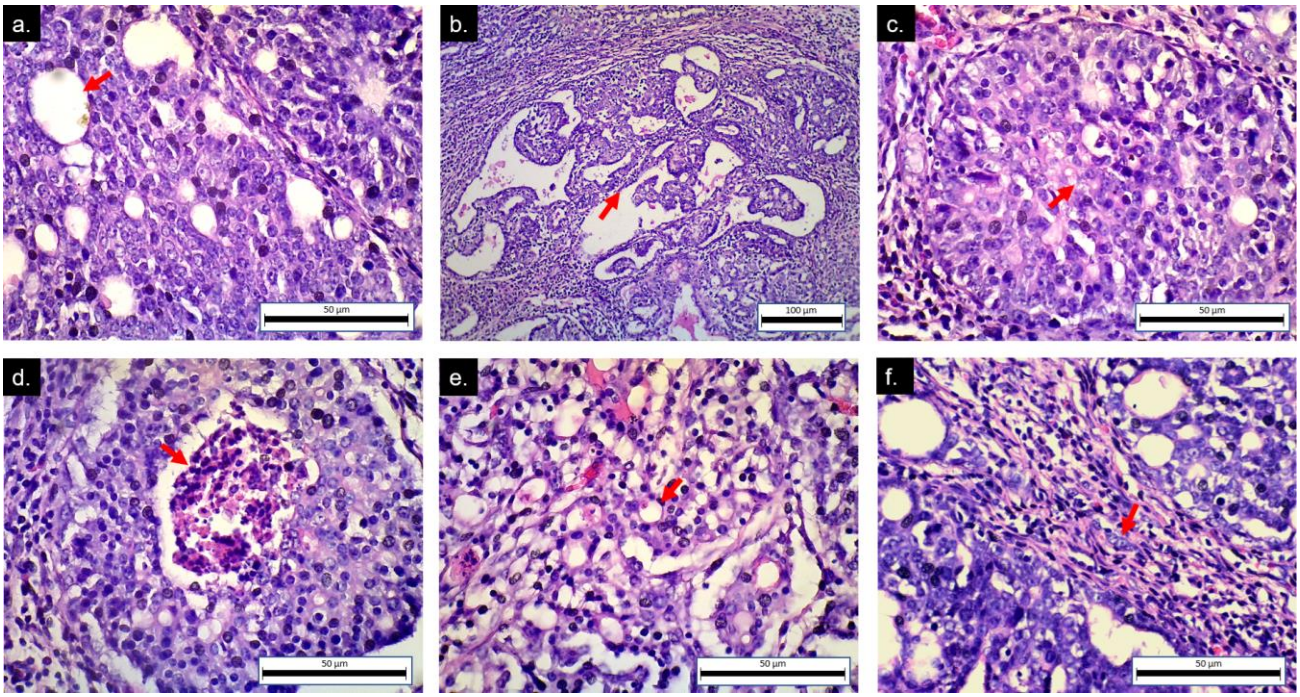

Figure 1. The mammary gland tumors induced by DMBA (7,12-dimethylbenz[a]anthracene) in rats. **a**: Cribriform ductal carcinoma *in situ* (DCIS), showing the proliferation of neoplastic cells with punched-out spaces within ductus (arrow) **b**: Papillary DCIS, where neoplastic cells form papillary structures with fibrovascular core within the ductus (arrow) **c**: Solid DCIS, characterized by a solid proliferation of neoplastic cells within the ductus (arrow) **d**: Comedo DCIS, marked with proliferation of neoplastic cells with central necrosis (arrow) **e**: Lipid Rich Carcinoma, marked with abundant lipid vacuoles within tumor cells (arrow) **f**: Invasive Cribriform Carcinoma, demonstrating cribriform carcinoma with neoplastic cells invading the stroma (arrow).

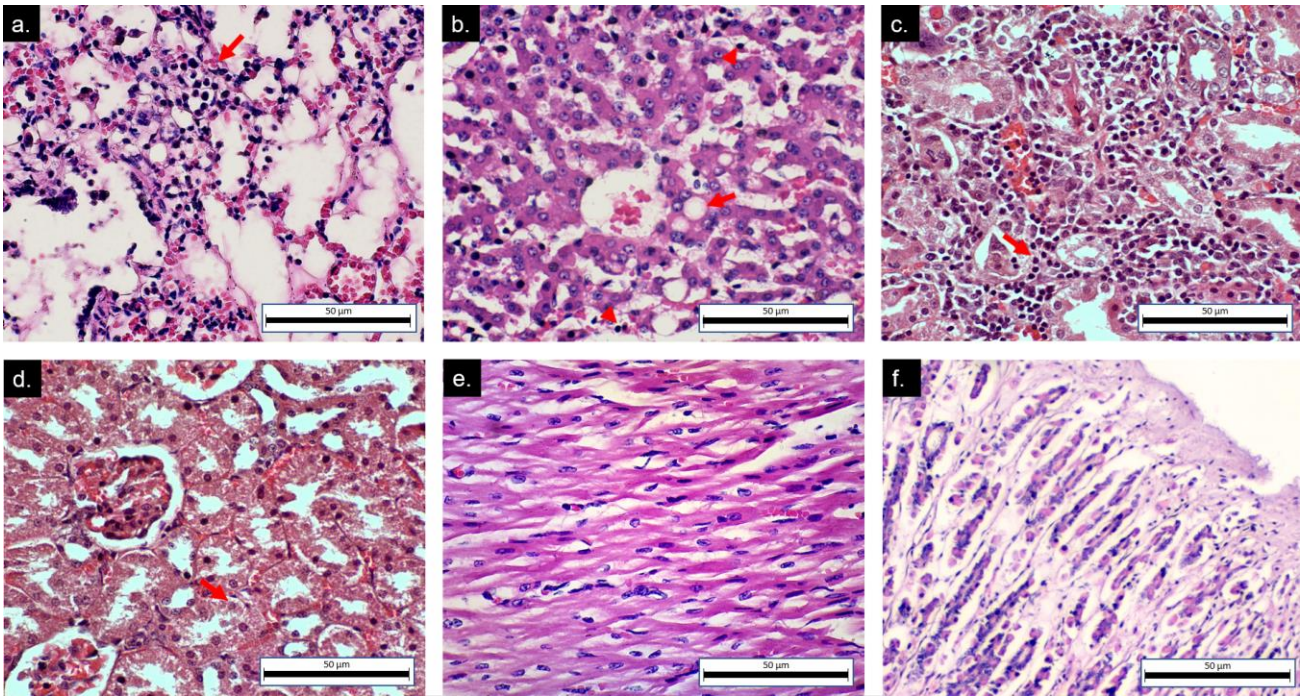

Figure 2. The internal organs of rats following subcutaneous administration of DMBA. **a**: Lungs, **b:** Liver, **c, d**: Kidney, **e**: Heart, and **f**: Stomach. The lungs (a) exhibit mild infiltration of mononuclear cells in the alveolar septa (arrow). The liver (b) shows vacuolar degeneration of hepatocytes (arrow) and mild inflammation (arrowhead). The kidney (c) exhibits tubular epithelial degeneration and mononuclear cell infiltration (arrow). The heart and stomach appear to be unremarkable.

DISCUSSION

The DMBA, a well-known member of the PAHs family, has been extensively studied through various administration routes to induce mammary cancer. The subcutaneous route, which directly targets the mammary fat pad, has demonstrated efficacy in producing localized tumors with minimal off-target effects. As reported by Barros et al. (2004), the oral administration of DMBA in rats resulted in an average of 4.9 nodules per animal, with a range of 1-15 nodules located at various sites. Similarly, Costa et al. (2020) reported that the induction of mammary cancer via oral administration of DMBA resulted in an average of 4.7 nodules per animal, with an average of 3.67 invasive tumors per animal. In contrast, in the current study, DMBA was injected subcutaneously into the mammary fat pad, which resulted in consistent tumor formation at the targeted site with minimal impact on other organs. This method produced a single tumor nodule per animal, making it a suitable model for studying localized cancer treatments like intratumoral therapy.

In the present study, increased DMBA dosage is associated with higher tumor incidence. However, there were no significant differences in tumor grades were observed among the various dosing groups, suggesting that while the subcutaneous method effectively induces localized tumors, it may not promote the progression to more aggressive cancer forms within the three-month observation period. The finding is consistent with Kubatka et al. (2002), who also demonstrated a dose-dependent relationship between DMBA dosage and tumor incidence, albeit with different administration routes and dosing regimens. Kubatka et al. (2002) found that administering repeated doses via oral induced a higher incidence of tumors compared to a single dose. The rarity of metastasis in DMBA-induced cancers suggests that a longer observation period might be necessary to observe metastatic progression (Abba et al., 2016).

In the present study, the histopathological analysis revealed a diverse range of tumor types, including papillary DCIS, solid DCIS, cribriform DCIS, comedo DCIS, lipid-rich carcinoma, and invasive cribriform carcinoma. Notably, cribriform DCIS was the most frequently observed type. The occurrence of lipid-rich carcinoma is consistent with the findings of Fidianingsih et al. (2022), who identified this rare tumor type following oral DMBA administration. Chemical induction of cancer generally results in a broader spectrum of tumor types compared to tumor transplantation in immunocompetent mice. Nevertheless, it effectively mimics the multistep carcinogenesis process observed in human breast cancer (Costa et al., 2020).

Molecularly, breast cancer is classified into distinct subtypes, including luminal A, luminal B, HER2-positive, and triple-negative based on the expression of specific biomarkers. The classification is based on the expression of estrogen receptor (ER), progesterone receptor (PR), HER2, and Ki67 (do Nascimento and Otoni, 2020; Fidianingsih et al., 2022). Given that luminal subtypes are the most common in humans, further research is required to ascertain whether the

subcutaneous DMBA model accurately reflects human breast cancer subtypes by examining these proteins and receptor expression through immunohistochemistry.

The present study revealed that organs, including the lungs, liver, and kidneys exhibit indications of degeneration and inflammation. However, no evidence of necrosis, metastasis, or neoplasia was observed. Reactive metabolites formed during DMBA metabolism, such as 7,12-DMBA-3,4-diol-1,2-epoxides have been demonstrated to cause tissue damage (Batcioglu et al., 2012). The observed inflammation in certain organs is likely attributable to DMBA, which has been linked to an inflammatory response through increased pro-inflammatory cytokines (Youssef et al., 2022).

According to Tedardi et al. (2013), the oral administration of DMBA in Balb/c mice not only induces breast cancer but also leads to tumor development in other tissues, including the skin (1.43%), stomach (7.14%), lymphoid tissues (11.43%), and lungs (17.14%). Similarly, Dias et al. (1999) found that oral administration of DMBA in female Sprague Dawley rats can induce tumors beyond breast cancer, including benign tumors of the salivary glands (3.57%), ovary (5.35%), liver (1.78%), and epiploon (0.89%). They also documented the emergence of malignant tumors in the salivary gland (1.78%), lymphatic system (1.78%), eye (0.89%), lungs (0.89%), and pancreas (0.89%).

Batcioglu et al. (2012) found that intraperitoneal administration of DMBA can result in the formation of necrotic areas within the liver and the infiltration of inflammatory cells. Additionally, Allam et al. (2023) reported that oral DMBA administration can lead to hepatocyte vacuolization, apoptosis, and renal tubular epithelial degeneration. The administration of DMBA orally for the induction of mammary cancer induction has been associated with pathological changes in the stomach, including mucosal and submucosal hemorrhage, epithelial cell hyperplasia, necrosis, and the development of precancerous lesions (Budi and Widyarini, 2010). Furthermore, there have been reports of its ability to induce adrenal toxicity (Costa et al., 2020).

The lesions observed in the study were not severe, indicating that the subcutaneous administration of DMBA minimized the systemic impact while effectively inducing localized breast cancer.

CONCLUSION

The current study demonstrated that subcutaneous administration of DMBA at 80 mg/kg BW in the mammary fat pad of Sprague Dawley rats effectively induced mammary cancer with varying tumor types based on the dosing regimen. Tumors were observed to consistently arise from the injection site with no significant differences in tumor grade across treatments. Additionally, examination of internal organs, such as the lungs, heart, kidneys, and stomach revealed no evidence of tumor development or signs of toxicity. It is noteworthy that no mortality was observed during the study period. Further research is required to ascertain the molecular type of mammary cancer through immunohistochemical staining for Ki67, ER, PR, and HER2.

DECLARATIONS

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Authors' contributions

Palagan Senopati Sewoyo was responsible for the conceptualization, writing of the original draft, investigation, methodology, formal analysis, and visualization. Ni Luh Lasmi Purwanti contributed to the investigation, project administration, and funding acquisition. Muhammad Munawaroh handled the investigation, data curation, and funding acquisition. I Made Kardena contribute to supervision, validation, formal analysis, and writing of the review and editing. I Nyoman Mantik Astawa provided resources, supervision, writing of the review and editing, and validation. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that no competing interests exist.

Ethical considerations

All authors have diligently reviewed the manuscript for potential ethical issues, including plagiarism, research misconduct, data fabrication or falsification, and redundant publication.

Availability of data and materials

All data generated during this research are pertinent and have been included in the published article. For further information or inquiries, please contact the corresponding author.

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